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The Initiation, Multiplication, and Cryopreservation of Fraser Fir (*Abies fraseri* [Pursh] Poir.) Embryogenic Tissue for Somatic Embryogenesis

Abstract

Fraser fir (*Abies fraseri* [Pursh] Poir.) is a coniferous tree native to the southern Appalachian Mountains in the United States. Due to its restricted native range in a high-elevation habitat and long reproductive process, the forces of anthropogenic global climate change and invasive pests have made this species vulnerable to extinction (Conifer Specialist Group 1998). Research on ways to propagate mass numbers of conifers like the Fraser fir and restore forest productivity includes clonal propagation through somatic embryogenesis. Such research is critical to help ensure the survival of this species for both environmental and economic reasons. Fraser fir is the most popular Christmas tree in the United States and the primary Christmas tree species grown in North Carolina, where Christmas tree sales alone brought in a revenue of over $75 million dollars in 2011 (NCDA 2012). To explore potential methods of increasing embryogenic tissue initiation and growth, embryogenic tissue initiation and capture media were supplemented with the redox chemical sodium thiosulfate (158.09 mg/L) and were compared to control media. Although the redox medium yielded a higher average percent initiation (29.3% versus 26.9%), the results were not statistically significant ($p > 0.05$). To assess the effects of toxic carbohydrate hydrolysis products in autoclaved media, growth of embryogenic tissue was recorded for capture media with autoclaved sucrose and compared to the growth of tissue on media with filter-sterilized sucrose. The non-significant results suggest that filter-sterilization of sucrose is not necessary and does not inhibit embryonic tissue proliferation. High-mass initiations were selected for cryopreservation and were analyzed for new growth after removal from cryogenic storage. Ongoing research includes production of somatic embryos from designated high-yielding cultures removed from cryostorage, propagation of those cultures on maturation media, and germination of normal somatic embryos on germination media to effectively create highly efficient protocols for the somatic embryogenesis of Fraser fir.

Introduction

The Fraser fir is a foundation species in its native Southern Appalachian spruce-fir forests. It is a high-altitude tree whose population distribution is highly limited. Fraser fir populations are under further stress from biotic sources, such as invasive pests, and abiotic sources, particularly anthropogenic activity and its incurred global climate change (McManamay et al. 2011). Such factors in addition to the species' poor regeneration have contributed to its national classification as vulnerable species (Conifer Specialist Group 1998). This is not only a problem in regards to the ecological stability of the spruce-fir forests in the southern Appalachian Mountains, but Fraser fir is a popular Christmas tree

and has high economic value.

It is therefore of particular interest to conserve and facilitate regeneration of these trees through alternative methods, such as clonal propagation by somatic embryogenesis (Kim et al. 2009). The process of somatic embryogenesis (SE) centers around embryo formation from somatic tissue, and one methodology to achieve successful SE begins with embryo excision from the female gametophyte and the placement of the embryo along with the cut megagametophyte ontosolid media to allow for development of embryogenic tissue (Cairney and Pullman 2007).

Research on clonal propagation through somatic embryogenesis includes particular focus on many coniferous species. SE has proven to be effective with respect to several species, including *Pinus taeda*, or loblolly pine (Pullman et al. 2006), and even in hybrids of the *Abies* genus (Salajova et al. 1996). Current research in this area is not only aiming to propagate large numbers of economically important trees, but is also focused on the production of high-quality stock for nurseries and the development of storage protocols like cryopreservation (Kim et al. 2009).

The cryopreservation of embryogenic tissue plays an important role for the conservation of coniferous species in addition to facilitating the process of somatic embryogenesis. Transferring embryogenic tissue into cryostorage allows for the development of a large bank of different genotypes that can later be used as planting stock. Also, tissue decline is a recurring problem for species like *A. fraseri* because embryogenic tissue viability can begin to decline after approximately a year of storage on maintenance media. Tissue decline can be prevented by storage of that tissue in liquid nitrogen. Therefore the utilization of cryopreservation allows for the continued use of embryogenic tissue for somatic embryogenesis long after a year past original tissue initiation.

Effective protocols for the clonal propagation of Fraser fir through induced somatic embryogenesis have been established, including methods to proliferate embryo suspensor masses and maturation media components that have resulted in successful development of somatic embryos (Guevin et al. 1997). Additional studies have been published describing suitable germination media and germination protocols for Fraser fir somatic embryos (Kim et al. 2009). However, much remains to be studied in the arena of conifer SE, particularly with regards to developing Fraser fir somatic embryos from cultures taken directly from cryopreservation in liquid nitrogen. Exploring the efficacy of tissue growth following removal from cryostorage is an integral part of forming long-term cryopreservation methods capable of allowing proper SE of tissue cultures despite years in storage. In the wake of increasing environmental pollution and limited population distribution, efforts to establish highly efficient SE and cryostorage protocols for species like Fraser fir are under way.

Materials and Methods

Plant material and the effect of sodium thiosulfate on Fraser fir embryogenic tissue initiation

Four Fraser fir seed crosses were shipped to the Institute of Paper Science and

Technology at Georgia Tech from the Department of Forestry and Environmental Resources at N.C. State University. These seeds were rated by morphological stage, with three seed crosses (24, 11, and 51) chosen for plating on initiation media. Based on previous research on the facilitative effects of redox chemicals on initiation in other conifers such as loblolly pine (Pullman et al. 2009), we tested to see whether the addition of a redox chemical, 1 mM sodium thiosulfate, to initiation medium 2568 would promote Fraser fir initiation and ultimately result in greater initiation percentage in the three crosses.

The components of the control initiation medium 2568 and initiation medium 2874 are listed in Table 1. All the components were added together, with the exception of the filter-sterilized stock solutions (L-glutamine and brassinolide for medium 2568, L-glutamine, brassinolide, and sodium thiosulfate for medium 2874) and Gelrite. The pH was set at 5.7 using KOH and/or HCl, the Gelrite was added, and the media were autoclaved for 20 minutes at 121°C. After autoclaving, the media were allowed to cool for approximately ten minutes before the addition of the filter-sterilized ingredients. Seven mL of the media were poured into each well of Costar no. 3506 6-Well Culture Cluster Plates. The plates were wrapped twice with Parafilm and stored in the dark at approximately 25±2°C.

After the initiation media were prepared, the seed crosses were sterilized, cut open, and the megagametophyte was dissected in half. The embryos were then excised, and the excised embryo and cut megagametophyte were placed surface down onto the media 2568 and 2874 initiation plates. Approximately 100 embryos were plated on both media for crosses 24 and 11, while approximately 66 embryos were plated on both media for cross 51. The plates were then wrapped with two layers of Parafilm and stored in the dark at 25±2°C.

The explants were evaluated under a microscope approximately 2.5 months later for the development of embryogenic tissue. After rating, the initiation percentages of the 90 plates were calculated. The initiation percentage data and arcsine-transformed initiation percentage data were tested for statistical significance through analysis of variance (ANOVA) with an alpha value of 0.05.

Capture of embryogenic tissue on media with autoclaved sucrose or filter-sterilized sucrose

After inspecting the resulting initiations for embryonic tissue growth, the 14 initiations with particularly accelerated development were chosen for a trial exploring the effect of filter-sterilized and autoclaved (not filter-sterilized) sucrose in capture media. Research has been done on the negative effects of oxygen consumption induced by the hydrolysis of autoclaved carbohydrates in plant media (Rédei 1973), therefore we tested for the effects of autoclaved sucrose on the embryogenic tissue growth of Fraser fir.

Media 2226 and 2885 were prepared in similar fashion to the initiation media, except that medium 2226 had L-glutamine and sucrose filter-sterilized while medium 2885 only had one filter-sterilized stock solution (L-glutamine). Seven mL of each media were poured into 14 individual 60x15 mm Petri dishes. Each of the 14 initiations was split into two approximately equal masses of embryogenic tissue and transferred to the capture media. The Petri dishes were wrapped twice with Parafilm and stored in the

Table 1. Components of initiation, capture, cryostorage, maturation, and germination media

Components	Media and component amounts (mg/L)							
	2568	2874	2226	2885	2905	2921	1891	397
KNO_3	-	-	-	-	-	-	-	1,170
NH_4NO_3	-	-	-	-	-	-	-	206.3
K_2HPO_4	87.1	87.1	340	340	340	340	340	85
$CaCl_2•2H_2O$	-	-	-	-	-	-	-	220
$CaSO_4•2H_2O$	37.8	37.8	37.8	37.8	37.8	37.8	37.8	-
$MgSO_4•7H_2O$	394.2	394.2	394	394	394	394	394	185.5
KI	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.415
H_3BO_3	2.48	2.48	2.48	2.48	2.48	2.48	2.48	3.1
H_3PO_4	289.82	289.82	667.4	667.4	667.4	667.4	599	-
$MnSO_4•H_2O$	18.6	18.6	18.6	18.6	18.6	18.6	18.6	8.45
$ZnSO_4•7H_2O$	5.76	5.76	18.6	18.6	18.6	18.6	5.76	4.3
$Na_2MoO_4•2H_2O$	0.103	0.103	0.103	0.103	0.103	0.103	0.103	0.125
$CuSO_4•5H_2O$	3.75	3.75	3.75	3.75	3.75	3.75	3.75	0.25
$CoCl_2•6H_2O$	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.0125
$NiCl_2•6H_2O$	1.188	1.188	1.188	1.188	1.188	1.188	1.188	-
$FeSO_4•7H_2O$	16.68	16.68	16.68	16.68	16.68	16.68	16.68	13.93
Na_2EDTA	-	-	-	-	-	-	-	18.65
myo-Inositol	1,000	1,000	1,000	1,000	1,000	1,000	1,000	100
Sucrose	10,000	10,000	10,000[1]	10,000	10,000	10,000	-	20,000
Maltose	-	-	-	-	-	-	40,000	-
$C_{12}H_{10}Mg_3O_{14}•9H_2O$	266	266	266	266	266	266	266	-
Sodium thiosulfate[1]	-	158.09	-	-	158.09	-	-	-
L-Glutamine[1]	2,000	2,000	2,000	2,000	2,000	2,000	2,000	-
Thiamine•HCl	1	1	1	1	1	1	1	1.0
Pyridoxine•HCl	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.2
Glycine	-	-	-	-	-	-	-	2.0
ABA	1	-	-	-	-	-	5[1]	-
d-xylose	-	100	-	-	-	-	-	-
BAP	1	1	1.1	1.1	1.1	1.1	-	-
Brassinolide[1]	0.048	0.048	-	-	-	-	-	-
Paclabutrasol	0.33	0.33	-	-	-	-	-	-
Activated charcoal	-	-	-	-	-	-	-	2,500
Agar	-	-	-	-	-	-	-	8,000
Gelrite	3,000	3,000	3,000	3,000	3,000	-	3,000	-
Sorbitol	-	-	-	-	-	72,868	-	-
PEG 8,000	-	-	-	-	-	-	100,000	-
pH	5.7	5.7	5.7	5.7	5.7	5.7	5.7	5.7

[1] Filter-sterilized and added to cooled, autoclaved media.

darkness at 25±2°C for three weeks. At the end of this three week subculture, the masses of the initiations were recorded and the growth factors were calculated by dividing the final tissue masses by the initial tissue masses. These data were then subjected to ANOVA analysis for determination of statistical significance given a 95% confidence interval.

Effect of sodium thiosulfate on capture of embryogenic tissue and culture maintenance

After testing the effect of the redox chemical sodium thiosulfate on Fraser fir initiation percentage, our next step was to determine its effect on embryogenic tissue growth on capture media. Media 2885 and 2905 were prepared, with the two treatments differing only by the presence of filter-sterilized sodium thiosulfate in medium 2905. For this experiment, 157 genotypes from the initiation experiment were used. All the embryogenic tissue from each initiation was transferred to a 7 mL Petri dish, alternating between the two media and recording the initial mass of the tissue. The cultures were wrapped in Parafilm and stored in the dark room for six weeks, at which point they were weighed and transferred onto new maintenance medium (either 2885 or 2905). Six-week transfers were continued for culture maintenance. Their weights after each six-week period were recorded, used to calculate growth factors, and analyzed by ANOVA.

Embryogenic tissue cryopreservation

The genotypes with the greatest mass of embryogenic tissue from 2885 and 2905 maintenance media were selected for cryogenic storage, and eight cultures with tissue masses ranging from 1-2 g were weighed out. Each gram of tissue was combined with 1.5 mL 0.4M sorbitol medium in a beaker, sealed with Parafilm, and put on a shaker for the night. The next day, the beakers were put in an ice bath, and filter-sterilized dimethyl sulfoxide (DMSO) was measured out at a ratio of 150µL to 1 gram of tissue. One fifth of this DMSO was then added very gradually over half an hour. Each cryogenic 2 mL Nalgene vial received 1 mL of this mixture. This was repeated for each of the eight selected genotypes. The vials were transferred to a controlled freezer and brought down to -35°C through increments of 0.3°C each minute. The vials were moved into cryogenic storage boxes and placed in liquid nitrogen. One vial of each genotype was removed from cryostorage three weeks later. The vials were taken out of the liquid nitrogen and put into an incubator set at 37°C for one minute (or longer if not fully thawed). Each vial was then carefully emptied onto a square of nylon on a Petri dish containing 20 mL of maintenance medium 2885. After an hour had passed, the cultures and nylon were put onto new media, sealed, and stored in a dark room. This last step was repeated eighteen hours later. These eight cultures were further analyzed for new growth after cryopreservation. A second round of cryostorage was later conducted with similar protocols with 10 genotypes from maintenance media with tissue masses ranging from 1 - 2 g, two of which were genotypes already in cryostorage from the first round of cryopreservation.

Maturation and formation of somatic embryos

After transfer onto maintenance media 2885, the cultures retrieved from cryostorage were analyzed for new tissue growth and viability after approximately eight weeks. Cultures that were not contaminated and showed definite growth were chosen for transfer onto maturation media 1891. The embryogenic tissue from each genotype was weighed and divided into pieces approximately ¼" in diameter, and those pieces were placed onto 1000x20 mm Petri disheseach containing 20 mL of maturation medium. The plates were sealed with Parafilm and stored in the darkness at 25±2°C for approximately three months with six week transfer periods.

Somatic embryo germination

After the three month maturation period, genotypes were assessed for the development of somatic embryos. The number of embryos per gram of embryogenic tissue and the average number of embryos per plate were calculated and recorded. Those with normal embryo shape were selected for germination testing. Normal embryos from maturation medium 1891 were transferred to Petri dishes containing 20 mL of germination medium 397, laying each embryo horizontally on the medium. The plates were wrapped with Parafilm and incubated in the dark for one week. Each plate was then removed from the dark and put under white fluorescent light (approximately 30 μmol photons $m^{-2}s^{-1}$) for two months. After those two months, the embryos were analyzed for successful germination on the basis that both a root and a shoot were present. Germination percentages were calculated for each genotype.

Results

Effect of sodium thiosulfate on Fraser fir embryogenic tissue initiation

The embryogenic tissue development of the 90 plates of explants was examined after six weeks to determine the number of initiation genotypes. The average initiation percentages by medium and seed cross are shown in Table 2. Medium 2568 yielded a 26.9% average initiation across the three seed lines, while medium 2874 yielded a higher average at 29.3% initiation. This is excluding the ten genotypes that were contaminated during the storage period. The *p*-value of 0.5414 for the effect of the two media on initiation percentage from ANOVA indicates that medium 2568 and medium 2874 did not result in significantly different initiation percentages. There is a higher initiation percentage seen for medium 2874 with sodium thiosulfate (Figure 1) despite lack of statistical significance. The initiation percentages varied by source cross, as well (33.4% for cross 11, 36.4% for cross 24, 14.5% for cross 51). There is no definite correlation seen in the crosses over the two treatments, and crosses 11 and 24 actually had higher initiation percentages on opposite media (Figure 2). The arcsine-transformed initiation percentages reinforced that the two media did not show statistically significant percent initiation results ($p = 0.7224$, Fig. 3, 4). These results suggest that while the sodium thiosulfate-supplemented medium did have a higher average percent initiation, the difference from the control medium was not great enough to be considered significant.

Table 2. Fraser fir initiation for three seed source crosses on control media and media containing sodium thiosulfate

Initiation medium	Sodium thiosulfate (mg/L)	Seed cross number and initiation percentage averages			
		11	24	51	Average % initiation
2568	0	26.0 ± 4.3	41.1 ± 4.3	13.6 ± 5.3	26.9 ± 2.7 a[1]
2874	158.09	40.8 ± 4.3	31.6 ± 4.3	15.5 ± 5.3	29.3± 2.7 a

[1] Different letters indicate significantly different values at the 95.0% confidence level through ANOVA.

Capture of embryogenic tissue on media with autoclaved sucrose or filter-sterilized sucrose

The growth of the 14 initiations on medium 2226 (containing filter-sterilized sucrose) was compared to the growth of the 14 genotypically identical initiations on medium 2885 (containing autoclaved sucrose). As shown in Table 3, medium 2226 resulted in an average growth factor of 9.3, and medium 2885 resulted in a very similar average (9.6). The ANOVA *p*-value of 0.9522 is extremely high, indicating that the difference in the average growth factors of the embryogenic tissue on the two treatment media was not statistically significant (Figure 5). This suggests that filter-sterilization of the sucrose is unnecessary in the preparation of Fraser fir capture media and results in no significant increase in embryogenic tissue growth. Only initiations from crosses 11 and 24 were tested in this experiment, with average growth factors of 16.7 and 2.2, respectively.

Effect of sodium thiosulfate on capture of embryogenic tissue

The remaining initiations were transferred to capture media 2885 and 2905 to test for the effect of the redox chemical sodium thiosulfate on initiation tissue growth. The cultures were weighed after six weeks and after twelve weeks, and the resulting average growth factors over the two six-week transfer periods are shown in Table 4. There was

Table 3. Fraser fir embryogenic tissue growth for two seed crosses on capture media with autoclaved sucrose and media with filter-sterilized sucrose

Capture media	Seed cross number and growth factor averages		
	11	24	Average growth factor
2226	15.8 ± 5.7	2.8 ± 0.6	9.3 ± 4.1 a[1]
2885	17.6 ± 8.7	1.6 ± 0.2	9.6 ± 4.1 a

[1] Different letters indicate significantly different values at the 95.0% confidence level through ANOVA.

Table 4. Growth of Fraser fir embryogenic tissue capture on control media and media containing sodium thiosulfate after twelve week transfer

Capture media	Seed cross number and growth factor averages			
	11	24	51	Average growth factor
2885	2.4 ± 0.5	2.7 ± 0.5	1.4 ± 1.0	2.2 ± 0.4 a[1]
2905	1.6 ± 0.5	2.2 ± 0.5	1.0 ± 1.0	1.6 ± 0.4 a

[1] Different letters indicate significantly different values at the 95.0% confidence level through ANOVA.

little variation in the growth factors seen amongst the two treatment media (average 2.2 for 2885, average 1.6 for 2905) for these two subculture periods. Based on ANOVA analysis of the growth of the genotypes over twelve weeks on maintenance medium, sodium thiosulfate does not appear to have a significant effect on embryogenic tissue growth ($p > 0.05$). Average growth factors by cross and interaction of cross and media type are shown in Figures 6 and 7.

Embryogenic tissue cryopreservation

Cultures were allowed eight weeks on maintenance medium 2885 following retrieval from cryostorage. Results of the first round of cryostorage are listed in Table 5. One vial was removed per genotype. Six genotypes demonstrated new growth after the eight weeks and were transferred onto maturation medium 1891. One genotype showed such little growth that successful embryo development was unlikely, therefore it was not transferred onto maturation media. One culture became contaminated shortly after removal. The genotypes and number of vials per genotype put into cryostorage for the second round are shown in Table 6. New tissue growth was assessed after approximately

Table 5. First round of cryostorage of Frasier fir embryogenic tissue

Genotype	Init. Year	Vials in LN	Vials out of LN	Vials left in LN	New growth after 8w	Rack	Box	Transferred to medium 1891
3	2012	3	1	2	Yes[1]	18	5	No
43	2012	2	1	1	Yes	18	5	Yes
79	2012	2	1	1	Yes	18	5	Yes
91	2012	4	1	3	Yes	18	5	Yes
99	2012	4	1	3	Yes	18	5	Yes
101	2012	2	1	1	n/a[2]	18	5	No
110	2012	4	1	3	Yes	18	5	Yes
112	2012	2	1	1	No	18	5	No

[1] Culture showed signs of new growth, but not enough to transfer onto maturation medium. Transferred onto new maintenance medium 2885.
[2] Culture became contaminated shortly after removal from cryostorage.

Table 6. Second round of cryostorage of Frasier fir embryogenic tissue

Genotype	Init. Year	Vials in LN	Vials out of LN	Vials left in LN	New growth after 8w	Rack	Box	Transferred to medium 1891
1	2012	2	1	1	No	18	5	No
6	2012	2	1	1	No[1]	18	5	No
24	2012	2	1	1	No	18	5	No
26	2012	2	1	1	No[1]	18	5	No
40	2012	4	1	3	No	18	5	No
70	2012	3	1	2	Yes	18	5	Yes
79	2012	2	1	1	Yes[1]	18	5	No
83	2012	2	1	1	Yes	18	5	Yes
85	2012	3	1	2	No[2]	18	5	No
91	2012	4	1	3	No[1]	18	5	No

[1] Culture became contaminated shortly after removal from cryostorage.
[2]Culture showed signs of new growth, but not enough to transfer onto maturation medium. Transferred onto new maintenance medium 2885.

eight weeks on retrieval medium 2885.

Maturation and formation of somatic embryos

Five genotypes from the first round of cryostorage were selected for transfer onto maturation medium 1891. After three months on maturation media, including one six week transfer, the genotypes were observed for the development of somatic embryos, with the number of cotyledonary embryos formed per genotype plate shown in Table 7. Two genotypes failed to develop any somatic embryos, and the three remaining genotypes averaged 7.8 embryos per gram of embryogenic tissue. The two genotypes from the second round of cryostorage were transferred onto medium 1891 and are currently in the three month maturation process. Figure 8 contains photographs taken of somatic embryos developing on maturation medium from the first round of cryostorage.

Somatic embryo germination

The embryos of the three genotypes from maturation media 1891 from the first

Table 7. Number of cotyledonary embryos formed after three months on medium 1891 from first round of cryostorage

Genotype	Mass (mg)	Number of cotyledonary embryos	Embryos/g of embryogenic tissue
43	170.0	3	17.65
79	154.0	0	0
91	3304.8	2	0.60518
99	779.1	4	5.134
110	718.3	0	0

round of cryostorage were transferred onto medium 397 to test for germination. After two months on germination medium, all the embryos from each of the three genotypes had developed shoots, but failed to develop roots (germination percentage = 0.0%).

Discussion

One genotype demonstrated a moderate embryonic yield per gram of embryogenic tissue. This is a promising starting point, for one of the main objectives of this study was to determine genotypes that yield large quantities of embryos and would therefore be suitable candidate genotypes for research on the mass propagation of Fraser fir. Additionally, of the three genotypes that successfully formed embryos, all the embryos developed shoots upon transfer to germination medium, but no roots emerged. Since germination is evidenced by the presence of both a root and a shoot, the three genotypes failed to germinate.

The experiments listed above represent adaptations of standard conifer initiation and maintenance procedures in an attempt to increase embryogenic tissue initiation and subsequent growth. Prior research of the effects of redox pairs in the seeds of loblolly pine (*Pinus taeda*) resulted in the conclusion that the addition of redox chemicals through media at key points in development can have positive effects on the somatic embryogenesis of loblolly pine and (Pullman et al. 2009). Because initiation and capture are important steps with respect to successful somatic embryogenesis, it was of interest to test the effects of a redox chemical on Fraser fir initiation and capture. Although this sodium thiosulfate did not have a significant effect on the initiation of the tested embryogenic tissues or the growth of the tissues on the capture media after four weeks, it is not suggestive that all redox chemicals are ineffective at enhancing somatic embryogenesis in this species. Analysis of other redox chemicals are logical extensions of this experiment, as well as supplementing growth media with sodium thiosulfate further along in development in the maturation process.

Although there is also lack of statistical significance with respect to the experiment testing the effects of filter-sterilized sucrose and autoclaved sucrose on embryogenic tissue growth, this serves to potentially improve the process of Fraser fir initiation growth. Previous research revealed that autoclaving carbohydrates such as fructose can lead to toxic degradation products that may inhibit the growth of embryogenic tissue on that media (Rédei 1973). The results from this experiment suggest that autoclaved sucrose had no significant effect, negative or positive, on the growth the embryogenic tissue, therefore filter-sterilization is not needed to achieve maximum growth. Because filter-sterilization of sucrose is more costly and time-consuming than simply autoclaving sucrose with the rest of the medium ingredients, this makes the process of media preparation more efficient.

Across the different stages of somatic embryogenesis, certain cultures had to be discarded due to contamination, particularly those on maintenance media after removal from cryostorage. This is evident in Tables 5 and 6, and the greater occurrence of contamination can possibly be explained by the use of media that had been made days before the actual transfer of the tissues and had been stored in a crisper until needed. The

storage of media in crispers can increase the chance of contamination because the Petri plates are not individually sealed and will breathe despite wrapping the crisper with Parafilm. For all future experiments, tissue will only be transferred onto freshly made media to decrease the likelihood of bacterial contamination.

Overall, the conclusions drawn from these series of experiments aid in our overarching goal of developing effective protocols for the clonal propagation of Fraser firs, from embryogenic tissue initiation to embryo development and germination.

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Figures

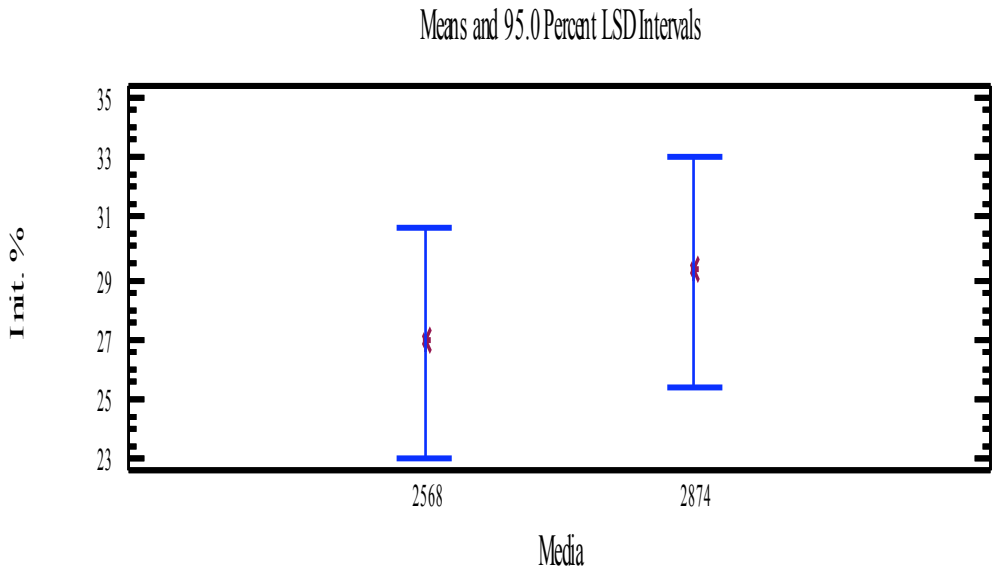

Figure 1. Means and 95.0 Percent Least Significant Difference intervals for initiation percentage by initiation medium. Medium 2568 resulted in 26.9 ± 2.7% average initiation, and medium 2874 resulted in 29.3± 2.7% average initiation. Average initiation percentages are nonsignificant across the two media ($p = 0.7224$).

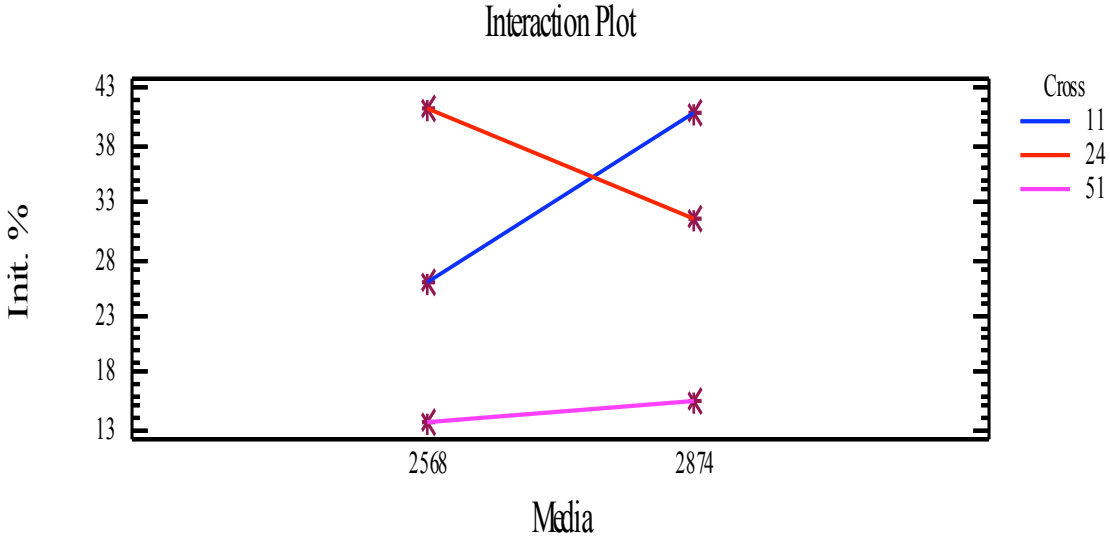

Figure 2. Interaction plot of three seed crosses and initiation media. No interaction evident for crosses 11 and 51. Possible interactions exists for cross 24 because it is not parallel to lines for crosses 11 and 51 ($p = 0.0211$).

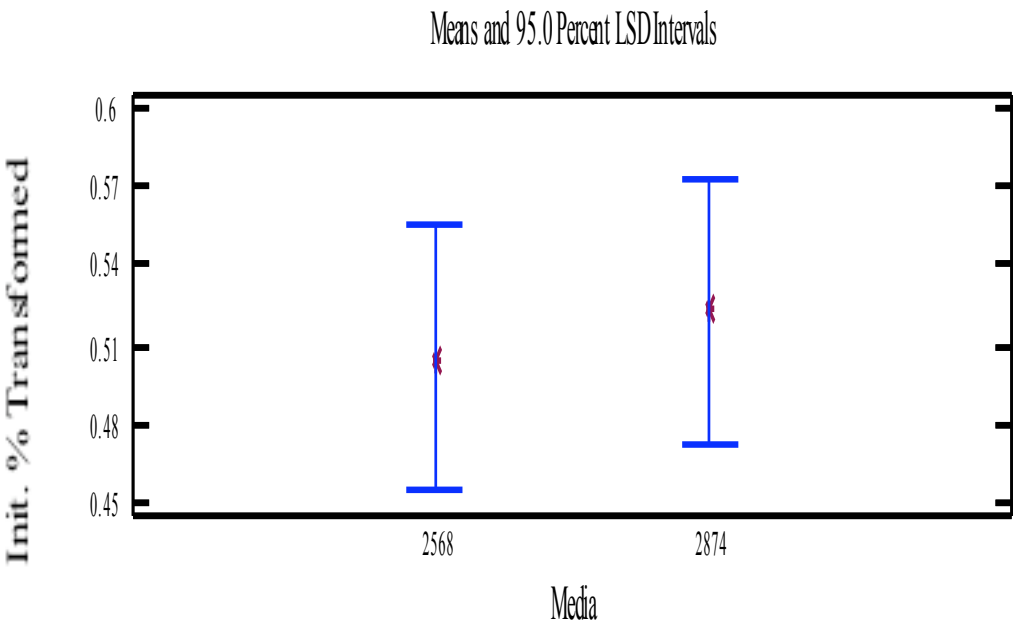

Figure 3. Means and 95.0 Percent Least Significant Difference intervals for transformed initiation percentage by initiation medium. Medium 2568 resulted in 0.506% transformed initiation average, and medium 2874 resulted in 0.523% transformed initiation average. Average transformed initiation percentages are nonsignificant across the two media ($p = 0.7224$).

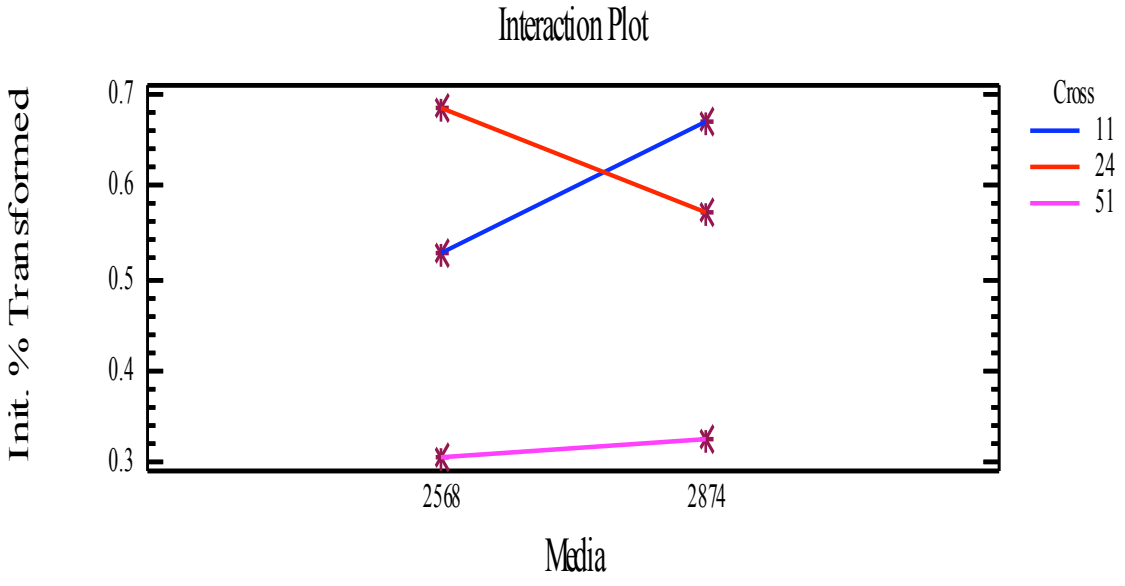

Figure 4. Interaction plot of three seed crosses and initiation media (transformed). No interaction evident for crosses 11 and 51. Possible interactions exists for cross 24 because it is not parallel to lines for crosses 11 and 51 ($p = 0.0724$).

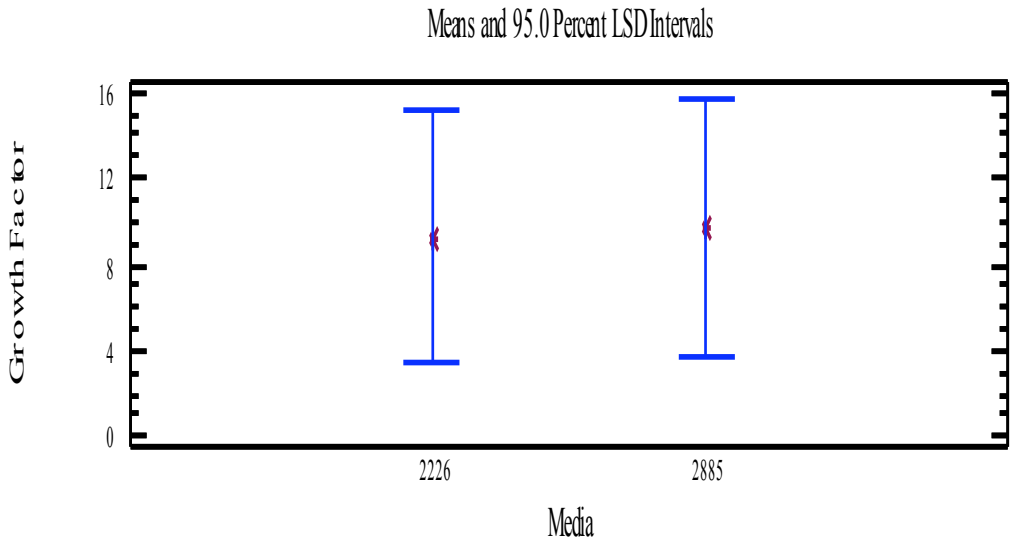

Figure 5. Means and 95.0 Percent Least Significant Difference intervals for growth factors by capture medium. Medium 2226 resulted in an average growth factor of 9.3 ± 4.1, and medium 2885 resulted in an average growth factor of 9.6 ± 4.1. Average growth factors are nonsignificant across the two media (p = 0.9522).

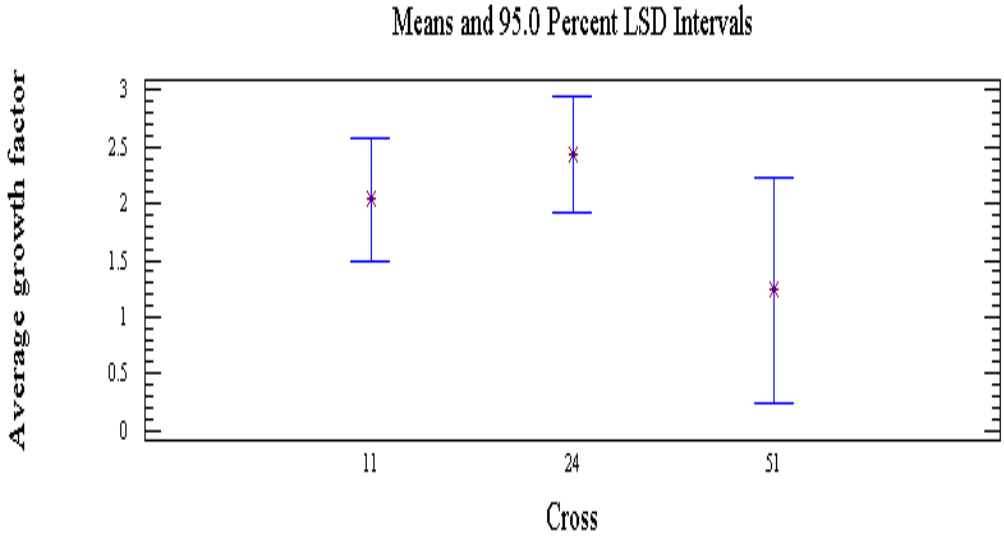

Figure 6. Means and 95.0 Percent Least Significant Difference intervals for average growth factors by cross. Cross 11 resulted in an average growth factor of 2.03, cross 24 resulted in an average growth factor of 2.4, and cross 51 resulted in an average growth factor of 1.23. Cross number is shown to be a nonsignificant main effect (p = 0.3211).

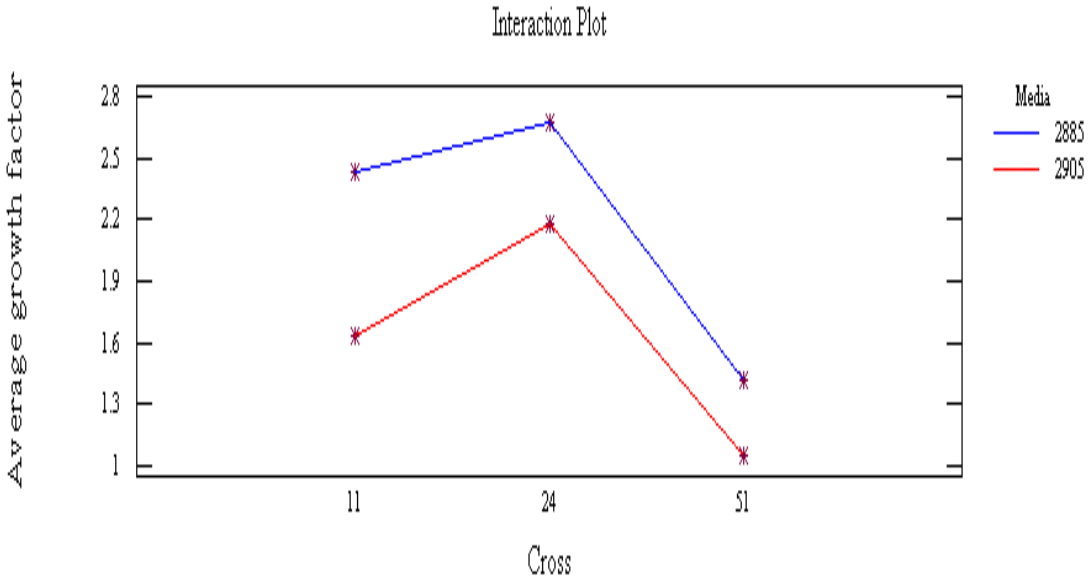

Figure 7. Interaction plot of three seed crosses and medium 2885 and medium 2905. Lines are parallel and therefore not indicative of an interaction effect between crosses and average growth factor (p = 0.9417).

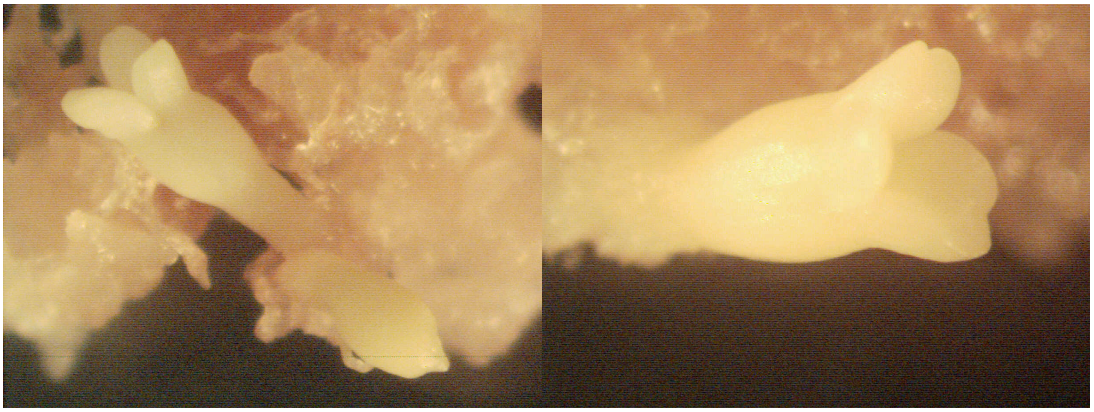

Figure 8. Fraser fir somatic embryos after approximately three months on mediaum1891.

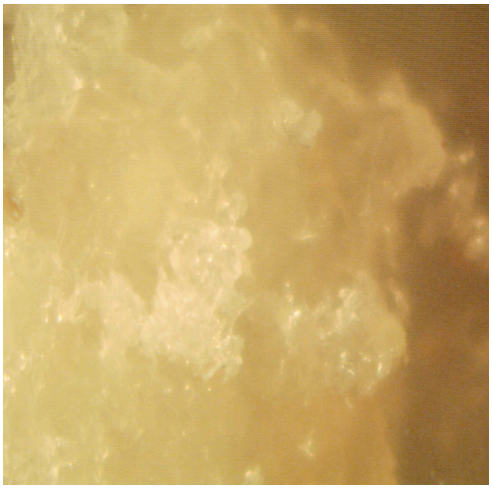

Figure 9. Fraser fir embryogenic tissue on maintenance medium 2885.